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Metabolism Clinical and Experimental 57 (2008) 691 – 697
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Effect of pitavastatin on type 2 diabetes mellitus nephropathy in KK-A^y/Ta mice

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Received 17 April 2007; accepted 22 January 2008

Abstract

It is generally considered that 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors (statins) have renoprotective effects via a pathway independent of their cholesterol-lowering cascade. In the kidneys of diabetic nephropathy, monomeric endothelial nitric oxide synthase (eNOS) is thought to be overexpressed; and its dimerization is suppressed. In the present study, we investigated the expression of eNOS and oxidative stress in type 2 diabetes mellitus KK-A^y/Ta mice treated with pitavastatin, one of the statins. The KK-A^y/Ta mice were divided into 3 groups and given pitavastatin intraperitoneally starting at 8 weeks of age for 8 weeks: pitavastatin 3 mg/(kg d) (n = 5), pitavastatin 10 mg/(kg d) (n = 5), and a control group (n = 10). The urinary albumin-creatinine ratio (ACR), urinary 8-hydroxy-2'-deoxyguanosine, body weight, fasting blood glucose, hemoglobin A_{1c}, total cholesterol, and triglyceride were measured; and the intraperitoneal glucose tolerance test was performed. The eNOS, nitrotyrosine, and p47 phox were evaluated by immunohistochemical analyses and/or Western blot analyses. Guanosine triphosphate cyclohydrolase 1 messenger RNA expression in the kidneys was evaluated using a real-time polymerase chain reaction assay. Pitavastatin improved the levels of urinary ACR and 8-hydroxy-2'-deoxyguanosine, intraperitoneal glucose tolerance test, and hemoglobin A_{1c}. Protein levels of monomeric eNOS, nitrotyrosine, and p47 phox in the kidneys were decreased in the pitavastatin-treated groups. Guanosine triphosphate cyclohydrolase 1 messenger RNA expression was significantly increased in the pitavastatin groups. There were no significant changes in body weight, levels of fasting blood glucose, serum total cholesterol, triglyceride, and blood pressure among all groups. Pitavastatin improved urinary ACR apparently because of suppression of eNOS uncoupling and its antioxidant effect in the kidneys of KK-A^y/Ta mice.

1. Introduction

The 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors (statins) have pleiotropic effects on cardiovascular, cerebrovascular, and microvascular diseases independent of their cholesterol-lowering effect [1-3]. Statins also have beneficial effects on kidney disease including diabetic nephropathy. Several studies have shown that statins prevented the progression of microalbuminuria and nephropathy in diabetes [4,5]. Although possible pathways of renoprotection, such as the Rho-dependent pathway, inhibition of macrophages, and platelet coagulation, were proposed, this effect of statins has not been fully clarified [6-9].

Nitric oxide (NO), known as a vasodilator and important factor for the prevention of arteriosclerosis and hypertension, is generated from vascular endothelial cells mainly by the endothelial nitric oxide synthase (eNOS) signal in the blood vessels [10,11]. In diabetic nephropathy, NO plays an important role in the maintenance of blood pressure by reducing renal vascular tone. Although generation of eNOS can be observed in all kinds of cells in the kidney, particularly strong expression is shown in the glomeruli. Endothelial nitric oxide synthase has 2 roles depending on whether it is in the monomeric or dimeric form. In the dimeric form, eNOS may act as NO generator. However, in the monomeric state, it produces superoxide (O_2^-) rather than NO [12]. Previous studies demonstrated that statins increased both eNOS and its cofactor, tetrahydrobiopterin (BH4), in diabetic endothelial cells. Furthermore, activity or gene expression of guanosine triphosphate cyclohydrolase 1 (GTPCH-1), the rate-limiting enzyme in BH4 synthesis, is

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doi:10.1016/j.metabol.2008.01.007

also up-regulated by statins [13-15]. In diabetic glomeruli, previous studies also indicated that the dimeric form of eNOS was decreased and the monomeric form was increased because of the decrease of BH4. Both NO activity and reactive oxygen species (ROS) production were increased. Such imbalance of NO/ROS may cause endothelial dysfunction of the kidney [1-3]. Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase is an important source of ROS production. Previous reports have noted that the components of NAD(P)H oxidase, such as p47 phox, p67 phox, and NOX4, were increased in diabetic kidneys [16,17].

In 1969, the KK-A^y/Ta mouse was established by Nishimura [18]. This mouse was produced by the transfer of the yellow obese gene (A^y allele) into the KK/Ta mouse. Because the diabetic feature in the KK-A^y/Ta mouse is more severe than that in the KK/Ta mouse, this mouse is widely used as an experimental model for type 2 diabetes mellitus [19]. The KK-A^y/Ta mouse spontaneously exhibits type 2 diabetes mellitus associated with hyperglycemia, glucose intolerance, hyperinsulinemia, obesity, and microalbuminuria. Renal lesions in the KK-A^y/Ta mouse closely resemble those in human diabetic nephropathy. Glomeruli of the KK-A^y/Ta mouse show diffuse-type hyperplasia of mesangial areas with mesangial cell proliferation, segmental sclerosis, overexpression of transforming growth factor–β1, and advanced glycation end product accumulation at 20 weeks of age [20-22]. We have reported that the KK-A^y/Ta mouse is a suitable model for the study of type 2 diabetes mellitus nephropathy in humans [21]. Furthermore, because mice have a naturally low cholesterol level and are generally unaffected by statin therapy, they are considered as good models for studying the pleiotropic effect of statins except the cholesterol-lowering effect.

In the present study, we investigated prevention of the development of type 2 diabetes mellitus nephropathy in KK-A^y/Ta mice treated with pitavastatin. We also investigated whether the renoprotective effect of statins is caused by a decrease of monomeric eNOS and whether the NAD(P)H oxidase components are decreased by statin treatment.

2. Materials and methods

2.1. Animals

Male diabetic KK-A^y/Ta Jcl mice (6 weeks of age) and C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). The mice were individually housed in plastic cages with free access to food (rodent pellet diet NMF; 348 kcal/100 g, containing 5.5% crude fat) and water throughout the experimental periods. All mice were maintained in the same room under conventional conditions with a regular 12-hour light/dark cycle and temperature controlled at 24°C ± 1°C.

2.2. Reagents and treatments

Pitavastatin was kindly provided by Kowa (Tokyo, Japan). Hyperglycemic KK-A^y/Ta mice were given pitavastatin in 0.5% carboxymethyl cellulose intraperitoneally starting at 8 weeks of age for 8 weeks. They were divided into 4 groups as follows: pitavastatin 3-mg/(kg d) treatment group (n = 5), pitavastatin 10-mg/(kg d) treatment group (n = 5), untreated control group (n = 10), and nondiabetic group (n = 5).

2.3. Biochemical characterization

The urinary albumin-creatinine ratio (ACR), body weight (BW), and hemoglobin A_{1c} (HbA_{1c}) were measured at 8, 12, and 16 weeks of age. The levels of serum total cholesterol, triglyceride, creatinine, and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) were measured at 16 weeks of age. Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT) in mice at 16 weeks of age.

Urinary samples were collected for 24 hours using a metabolic cage (mouse metabolic cage, CLEA Japan). Urinary albumin and creatinine samples were measured by immunoassay (DCA 2000 system; Bayer Diagnostics, Elkhart, IN) [19,21]. Glucose levels in blood obtained from the retroorbital sinus were measured using Glucocard (Kyoto Daiichi Kagaku, Kyoto, Japan). Hemoglobin A_{1c} was measured by immunoassay (DCA 2000 system, Bayer Diagnostics) [19,22]. Urinary 8-OHdG level was measured by enzyme-linked immunosorbent assay (Fushimi Pharmaceutical, Kagawa, Japan). Intraperitoneal glucose tolerance test was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the retroorbital sinus were measured using Glutest E (Kyoto Daiichi Kagaku) at 0 (fasting blood glucose level), 30, 60, and 120 minutes after the intraperitoneal glucose injection.

2.4. Immunohistochemical staining for eNOS, p47 phox, and nitrotyrosine

The mice were killed at 16 weeks of age. The kidneys were retrogradely perfused with saline via the abdominal aorta for 5 minutes at a pressure of about 150 mm Hg without prior flushing of the vasculature. Cryostat 3-μm kidney sections were air-dried for 10 minutes and then fixed in cold acetone for 10 minutes. To reduce the background, nonspecific binding was blocked by incubating with blocking solution (phosphate-buffered saline [pH 7.2] containing 2% bovine serum albumin, 2% fetal calf serum, and 0.2% fish gelatin) for 60 minutes. Endogenous peroxidase activity was inhibited by incubation with methanol containing 0.3% H_2O_2 for 10 minutes. The sections were then incubated with the primary antibody (Ab) diluted 1:100 in blocking solution at 4°C overnight. The primary Ab was polyclonal rabbit anti-eNOS Ab (BD

Transduction Laboratories, Lexington, KY), polyclonal rabbit anti-p47 phox Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal rabbit anti-nitrotyrosine Ab (Upstate Biotechnologies, Lake Placid, NY). Peroxidase activity was developed in 3,3-diaminobenzidine. Finally, Mayer hematoxylin was added as a counterstain. The secondary Ab was anti-rabbit Envision Plus polymer reagent (DAKO, Carpinteria, CA).

2.5. Quantitative messenger RNA analysis of GTPCH-1 by real-time polymerase chain reaction

The kidneys were dissected and snap-frozen in liquid nitrogen for total RNA extraction. The RNA was extracted with Trizol (Total RNA Isolation Reagent; Life Technologies, Rockville, MD). Complementary DNA was synthesized using random hexamers (Quantum RNA kit; Ambion, Austin, TX) and Superscript II RNaseH reverse transcriptase (Life Technologies). Primers and fluorogenic probes of GTPCH-1 were obtained from a commercial base (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA). The assay ID of GTPCH-1 was Mm00514993_m1 (Genbank ID: NM_008102.2). The complementary DNA obtained was further amplified using a real-time polymerase chain reaction (PCR) system (ABI Prism 7500 Real Time PCR System; Perkin-Elmer, Foster City, CA). Initial template concentration was derived from the cycle number at which the fluorescent signal crosses a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined based on the threshold cycles (Ct values). The PCR parameters were 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for 60 seconds.

2.6. Western blot analysis of eNOS, p47 phox, and nitrotyrosine

Portions of whole kidney samples were homogenized in phosphate-buffered saline with a Complete protease inhibitor cocktail tablet (Roche Diagnostics, Manheim, Germany). After centrifugation, the supernatants were used for Western blot analysis. Appropriate volumes of the supernatant (20 μg per lane) were mixed with an equal volume of sample buffer (312.5 mmol/L Tris-HCl [pH 6.8], 10% sodium dodecyl sulfate, 50% glycerol, 10% 2-mercaptoethanol, and 0.025% bromophenol blue) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% acrylamide gels. For immunoblot analysis of the monomer of eNOS, p47 phox, and nitrotyrosine, samples were heated at 95°C for 5 minutes before electrophoresis. The proteins were transferred by semidry electroblotting to polyvinylidene difluoride membranes for 60 minutes. The blots were then blocked and incubated with the first Abs for 60 minutes at room temperature. Next, the blots were incubated with the second Abs. The Ab was visualized using an enhanced chemiluminescence method (ECL Plus; Amersham Biosciences, Piscataway, NJ). The first Abs used in this study were as follows: 1:1000 dilution of rabbit polyclonal Ab to eNOS (BD Transduction Laboratories), 1:1000 dilution of rabbit polyclonal Ab to p47 phox (Santa Cruz Biotechnology), and 1:1000 dilution of rabbit polyclonal Ab to nitrotyrosine (Upstate Biotechnologies). Horseradish peroxidase–conjugated anti-rabbit second Abs (Jackson-Immunoresearch Laboratories, West Grove, PA) were used in this study. β-Actin (monoclonal anti–β-actin Ab; Sigma, St Louis, MO) was used for all membranes as an internal control, and signals on Western blots were quantified by densitometry and normalized relative to the β-actin signal using LAS-3000 image analysis software program (Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data were presented as mean ± standard error (SE). Comparison between groups was performed using analysis of variance with Bonferroni/Dunn post hoc testing. The relationships between the dose of pitavastatin and urine albumin, insulin sensitivity, and oxidative stress markers were evaluated using Pearson correlation. The relationships between the changes in urine albumin and insulin sensitivity, renal markers of oxidative stress, and eNOS were evaluated by regression analysis. Stepwise multiple regression analysis was performed to evaluate which parameter was closely related to ACR. P values of less than .05 were defined as statistically significant.

3. Results

3.1. Biochemical characterization

As shown in Fig. 1 and Table 1, the mean levels of ACR and HbA_{1c} at 16 weeks of age in both the 3- and 10-mg/(kg d) pitavastatin groups were significantly lower than those in the untreated group depending on the dosage of pitavastatin. The mean levels of HbA_{1c} in both the 3- and 10-mg/(kg d) groups were significantly lower than those in the untreated group. However, there were no statistically significant changes in the levels of HbA_{1c} between the 3- and

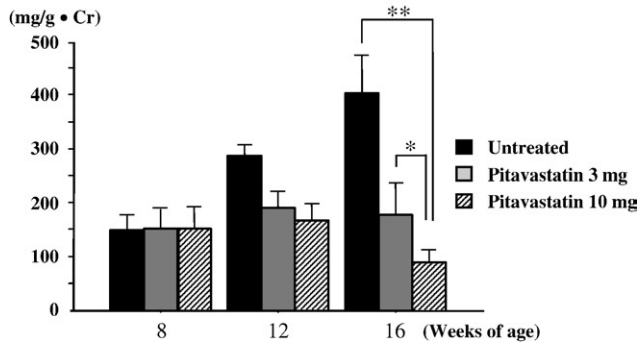

Fig. 1. Urinary ACR at each age (8, 12, and 16 weeks of age) in KK-A^y/Ta mice treated with pitavastatin. Data are expressed as means ± SE. *P < .01 vs untreated at 16 weeks. **P < .002 vs untreated.

Table 1
Biochemical profiles of KK-Ay/Ta mice treated with pitavastatin

		Nondiabetic	Untreated	Pitavastatin 3 mg	Pitavastatin 10 mg
BW (g)	8 w	21.8 ± 0.9	30.2 ± 0.5	29.4 ± 0.5	31.0 ± 0.7
	12 w	27.0 ± 0.4	39.1 ± 0.6	35.0 ± 1.0	37.7 ± 0.6
	16 w	29.7 ± 0.6	42.0 ± 0.8	37.1 ± 1.0	39.8 ± 1.0
Hb_{A1c} (%)	8 w	2.7 ± 0.1	4.6 ± 0.2	4.5 ± 0.2	4.5 ± 0.3
	12 w	3.2 ± 0.1	8.1 ± 0.8	6.7 ± 0.8	6.9 ± 0.5
	16 w	3.2 ± 0.1	9.2 ± 0.4	5.9 ± 0.5 **	5.5 ± 0.3 **
Fasting blood glucose (mg/dL)	8 w	52.6 ± 1.0	95.8 ± 5.6	105.6 ± 3.2	113.4 ± 9.0
	12 w	63.0 ± 0.9	97.0 ± 4.1	95.4 ± 7.3	84.2 ± 7.5
	16 w	108.3 ± 13.2	126.6 ± 16.2	98.4 ± 8.8	111.4 ± 12.7
Blood glucose after IPGTT (mg/dL)	30 min	386.3 ± 34.3	520.2 ± 19.6	479.0 ± 20.4	494.2 ± 17.0
	60 min	494.4 ± 70.8	531.4 ± 19.3	441.2 ± 27.2 *	451.4 ± 26.1 *
	120 min	204.4 ± 21.9	358.0 ± 53.3	223.2 ± 21.9 *	192.5 ± 17.3
Urinary 8-OHdG (ng/mg·Cr)		43.1 ± 12.9	84.2 ± 14.8	63.7 ± 21.2	28.8 ± 11.8 *
Serum creatinine (mg/dL)		0.28 ± 0.02	0.34 ± 0.02	0.34 ± 0.02	0.30 ± 0.00
Creatinine clearance (mL/min)		0.16 ± 0.04	0.27 ± 0.03	0.24 ± 0.15	0.24 ± 0.03
Serum total cholesterol (mg/dL)		102.4 ± 21.3	100.8 ± 5.8	115.8 ± 7.4	117.6 ± 7.6
Serum triglyceride (mg/dL)		113.3 ± 11.7	110.3 ± 3.5	101.0 ± 14.0	101.6 ± 5.3

Data are expressed as means ± SE.
* $P < .05$ vs untreated.
** $P < .0001$ vs untreated.

10-mg/(kg d) groups. There were no statistically significant changes in the levels of BW, fasting blood glucose, serum total cholesterol, triglyceride, creatinine, and creatinine clearance between the pitavastatin groups and the untreated group. In IPGTT, the mean levels of blood glucose at 60 and 120 minutes in the pitavastatin groups were significantly lower than those in the untreated group. The mean levels of urinary 8-OHdG at 16 weeks of age in both the 3- and 10-mg/(kg d) groups were significantly lower than those in the untreated group.

3.2. Immunohistochemical staining for eNOS, p47 phox, and nitrotyrosine

In immunohistochemical staining, eNOS and nitrotyrosine proteins were mainly localized in the glomerular

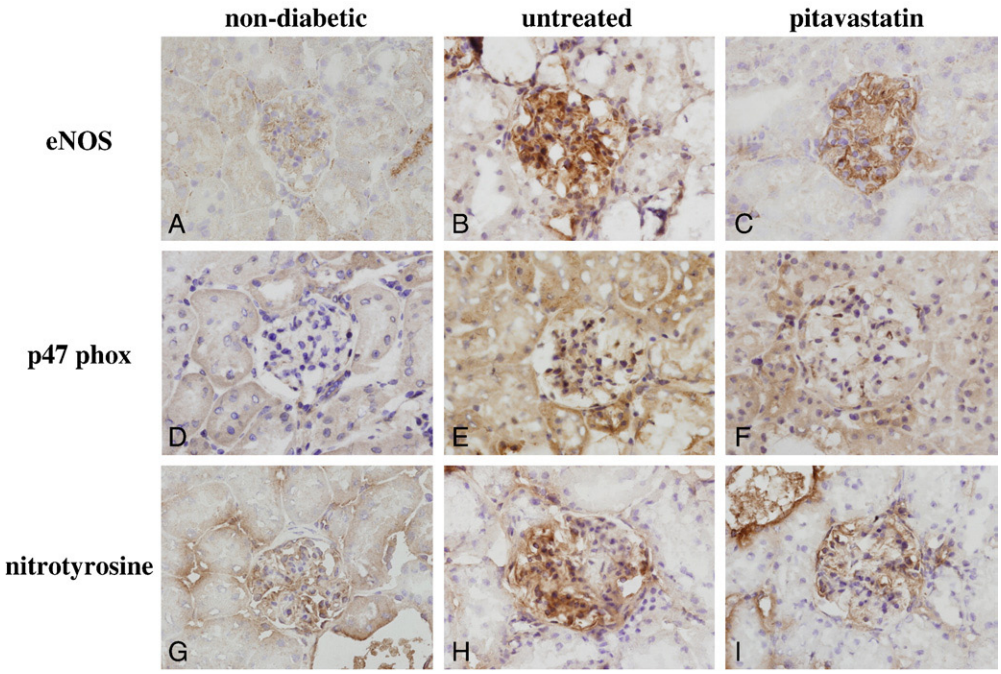

Fig. 2. Immunohistochemical staining for eNOS, p47 phox, and nitrotyrosine at 16 weeks of age in renal tissue sections treated with pitavastatin. A to C, Endothelial nitric oxide synthase staining. D to E, p47 phox staining. G to I, Nitrotyrosine staining. Original magnification ×400. Nondiabetic group (A, D, G), diabetic group (B, E, H), and pitavastatin-treated (10 mg/[kg d]) group (C, F, I).

mesangial areas. p47 phox protein was localized in the glomeruli, convoluted tubule, and interstitium. Accumulations of eNOS, p47 phox, and nitrotyrosine proteins in the diabetic groups were stained more intensely than those in the nondiabetic groups. Accumulations of these proteins at 16 weeks of age in the pitavastatin group decreased to the levels of the nondiabetic groups compared with those in the untreated group (Fig. 2).

3.3. Quantitative messenger RNA analyses of GTPCH-1 by real-time PCR

The messenger RNA (mRNA) levels of GTPCH-1 in both the 3- and 10-mg/(kg d) groups were significantly higher than those in the untreated group (Fig. 3).

3.4. Protein expression of monomeric eNOS, p47 phox, and nitrotyrosine by Western blot analysis

The protein expression levels of monomeric eNOS and p47 phox in the untreated group were significantly higher than those in the nondiabetic group. Pitavastatin treatment decreased the expression levels to the degree in the nondiabetic group (Fig. 4A, B). The expression level of nitrotyrosine in the untreated group was also significantly higher than that in the nondiabetic group and was decreased in the pitavastatin groups depending on dosage (Fig. 4C).

3.5. Statistical analysis

Evaluated by Pearson correlation coefficient, significant associations between the dosage of pitavastatin and the changes in urine albumin ($r = -0.719$, $P < .01$), HbA_{1c} ($r = -0.682$, $P < .01$), and the expression of renal nitrotyrosine protein ($r = -0.737$, $P < .05$) were observed. We could not find any significant associations between the dosage of pitavastatin and NAD(P)H oxidase p47 phox protein expression and eNOS protein expression, and between the dosage and urine 8-OHdG. Parameters statistically and significantly related to ACR were Hb_{A1c} ($r = 0.29$, $P = .021$), renal nitrotyrosine protein expression ($r = 0.412$, $P = .045$), renal p47 phox protein expression ($r = 0.495$, $P = .023$), and renal eNOS protein expression ($r = 0.492$, $P = .023$). Because these parameters might be closely correlated with each other, stepwise multiple regression analysis was performed. As a result, p47 phox protein expression ($P = .023$) was found to be significantly related to ACR ($R^2 = 0.432$).

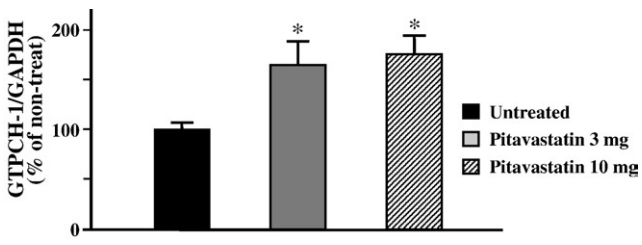

Fig. 3. Mean expressions of mRNA for GTPCH-1 in renal tissue by real-time PCR at 16 weeks of age. Data are expressed as means ± SE. *$P < .01$ vs untreated. **$P < .001$ vs untreated.

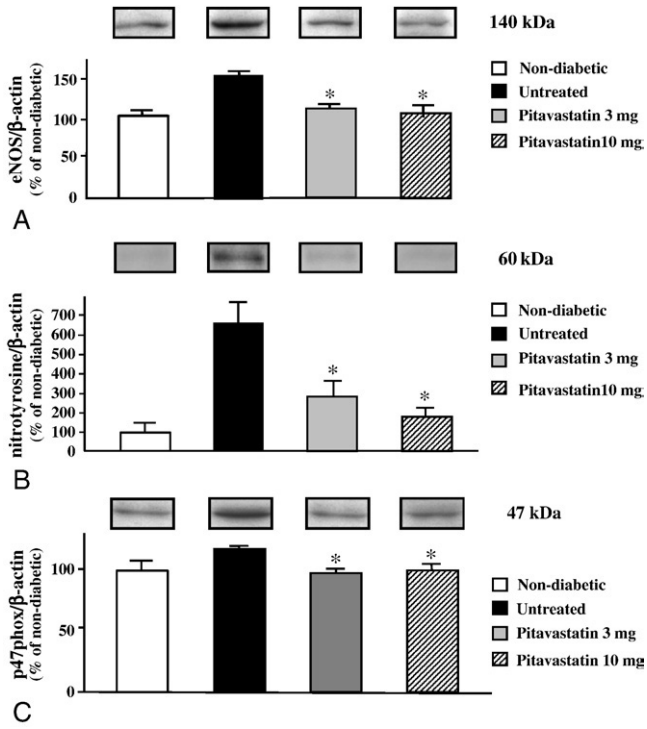

Fig. 4. Mean protein levels of eNOS, p47 phox, and nitrotyrosine at 16 weeks of age in KK-A^y/Ta mice treated with pitavastatin by Western blot analyses. A, Expression of monomeric eNOS. B, Expression of p47 phox. C, Expression of nitrotyrosine levels. Data are expressed as means ± SE. *$P < .01$ vs untreated.

4. Discussion

In the present study, we demonstrated that pitavastatin improves the levels of urinary ACR, urinary 8-OHdG, and insulin resistance in KK-A^y/Ta mice independent of its cholesterol-lowering effect. Furthermore, pitavastatin prevented accumulation of monomeric eNOS, nitrotyrosine, and p47 phox in the kidney tissues.

Although statins have been reported to have pleiotropic effects on cardiovascular, cerebrovascular, and microvascular disease independent of their cholesterol-lowering effects in several clinical investigations, evidence of renoprotection in humans has not been presented. Previous reports have shown the pleiotropic effects of statins, such as anti-inflammatory effects and antioxidative stress effects in vitro and in vivo [1,2]. However, the detailed inhibitory mechanism on the progression of diabetic nephropathy by pitavastatin is still obscure.

Mechanisms of improvement of urinary ACR by statin treatment have been proposed in some reports [3,4,23,24]. They stated that cerivastatin improved the urinary ACR of

diabetic rats by an anti-inflammatory effect and/or inhibition of macrophage recruitment and activation, and also by inhibition of transforming growth factor–β overexpression.

Previously, we suggested that oxidative stress and nitrotyrosine are related to progression of diabetic nephropathy [25]. *Oxidative stress* is defined as tissue injury induced by an increase of ROS such as the hydroxyl radical, superoxide anion, and hydrogen peroxide. Thus, oxidative stress is considered to be one of the factors involved in the development of diabetic complications [26,27]. Peroxynitrite nitrosylates the tyrosine residue on proteins and produces nitrotyrosine in diabetic nephropathy. In this study, ACR and the urinary excretion levels of 8-OHdG, a marker of oxidative DNA damage, were improved with suppression of nitrotyrosine in the diabetic kidney by pitavastatin treatment. Because the murine serum cholesterol levels are originally low, statins are believed not to affect their serum cholesterol level. In this study, pitavastatin affected neither the serum cholesterol levels (Table 1) nor the blood pressure (data not shown). Thus, we may be able to explain one of the mechanisms of the improvement of ACR as due to a decrease of oxidative stress. Furthermore, previous studies have reported that not only increases of nitrotyrosine and 8-OHdG but also increases of NAD(P)H oxidase were observed in diabetic kidneys. The NAD(P)H oxidase is composed of several subunits, and its translocation from cytoplasm to membrane is important in the production of ROS. Each NAD(P)H oxidase component, such as p47 phox, gp91 phox, p22 phox, and NOX4, has been shown to increase in the kidneys of diabetic rats [16,28]. Inhibition of these NAD(P)H oxidase components is thought to reduce oxidative stress. Kitada et al [17] reported that the inhibition of PKC-β, one of the isoforms of PKC, with ruboxistaurin inhibited the translocation of p47 phox and p67 phox from cytoplasm to membrane in diabetic nephropathy. Although we have not examined whether PKC and PKC-β are inhibited by statin treatment, previous articles have suggested that stains improved the PKC activation [8]. They revealed that high glucose levels induced PKC-dependent activation of the small GTP-binding protein Rac-1, a regulatory component of NAD(P)H oxidase. Therefore, it is reasonable to assume that statins decrease NAD(P)H oxidase because of the inhibition of PKC in diabetic kidneys.

Decrease of the level of monomeric eNOS was observed in the pitavastatin-treated group in this study. Endothelial nitric oxide synthase forms a dimer when there is sufficient BH4 present, but it forms a monomer in the absence of BH4. Satoh et al [12] reported that monomeric forms of eNOS were increased and dimeric forms of eNOS were decreased in diabetic glomeruli. They also demonstrated that the levels of p22 phox, p47 phox, and p67 phox were decreased by BH4 administration in diabetic rats. Guanosine triphosphate cyclohydrolase 1 is one of the essential rate-limiting enzymes of BH4. In the absence of GTPCH-1, biopterin is induced to produce the inactivated form, BH2, instead of BH4. Hattori et al [13] reported that statin treatment up-regulated GTPCH mRNA and eNOS mRNA in diabetic vascular endothelial cells in an in vitro study. Therefore, one of the possible mechanisms of improvement of ACR and renal oxidative stress by statins is as follows (Fig. 5). Isoprenoids, such as farnesyl pyrophosphate or geranylgeranyl pyrophosphate, are generated from HMG-CoA through mevalonate depletion. Isoprenoids inhibit the generation of eNOS and GTPCH-1, and they also increase NAD(P)H oxidase through inhibition of the Rho pathway and activation of Rac-1. Because pitavastatin inhibits HMG-CoA reductase and blocks synthesis of the isoprenoids, generation of NAD(P)H oxidase is inhibited and signals to generate eNOS are up-regulated. Furthermore, statins activate GTPCH-1 and lead to up-regulation of BH4, which is essential for eNOS to form a dimer. As a result, pitavastatin activates eNOS dimerization and enforces their stability through this cascade. As monomerization of eNOS that involves NO and ROS imbalance is decreased, oxidative stress is decreased. Moreover, up-regulated dimeric eNOS acts as an NO generator and may work against shear stress in the early stage of diabetic nephropathy.

Furthermore, we also found dose-dependent improvement of HbA_{1c} and blood glucose levels 60 and 120 minutes after IPGTT in this study. These results also suggest the mechanism of improvement of urinary ACR through the improvement of insulin resistance. Kureishi et al [29] reported that simvastatin and pravastatin led to a dose-dependent increase in phosphorylation of Akt/PKB, which is essential for the insulin signaling cascade as well as regulation of cell survival and stimulation of NO. Wong et al [30] reported that treatment with atorvastatin led to a dose-dependent improvement in whole-body insulin sensitivity in lean and fatty rats. Our results confirm their reports. Svensson and Eriksson [31] also discussed the relation between insulin resistance and microalbuminuria in the early stage of human diabetic nephropathy. They reported that insulin resistance might be both a cause and a consequence of diabetic

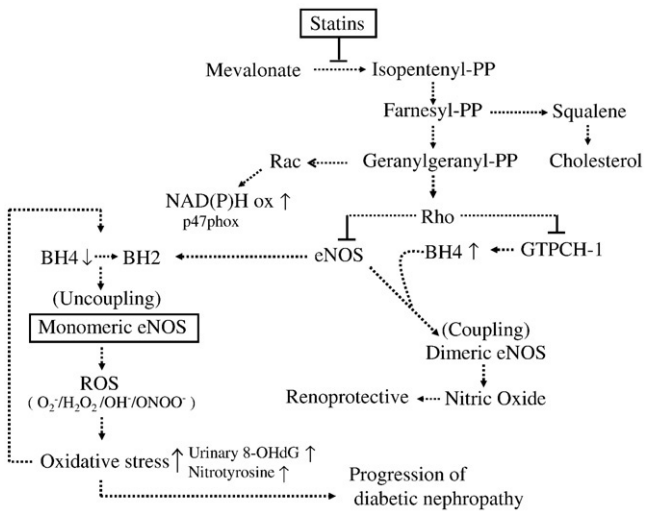

Fig. 5. Possible pathway of improvement of oxidative stress by statins.

nephropathy, altering the levels of hormones and adipokines. However, there still remain some problems in our study. First, improved glycemic control by pitavastatin could partly modify all the other parameters such as ACR and nitro-oxidative stress in the kidney. Furthermore, the mechanism of dose-dependent amelioration of insulin resistance by statins is not fully demonstrated; and the safety dose for humans with ameliorating insulin resistance has not been established. Further study will be needed about these problems.

In conclusion, it appears that pitavastatin improved not only urinary ACR but also HbA_{1c} and impaired glucose tolerance in KK-A^y/Ta mice, a spontaneous animal model for type 2 diabetes mellitus nephropathy, which might be because of the suppression of eNOS uncoupling and its antioxidant effects in diabetic kidneys.

Acknowledgment

We thank Kowa (Tokyo, Japan) for providing pitavastatin. We also want to thank Ms T Shibata and Mr K Yoshida for their skillful technical supports.

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